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Practical use of the Virtual Cell Based Assay: Simulation of repeated exposure experiments in liver cell lines

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ABSTRACT

The Virtual Cell Based Assay (VCBA) was applied to simulate the long-term (repeat dose) toxic effects of chemicals, including substances in cosmetics and personal care products. The presented model is an extension of the original VCBA for simulation of single exposure and is implemented in a KNIME workflow. This work illustrates the steps taken to simulate the repeated dose effects of two reference compounds, caffeine and amiodarone. Using caffeine, *in vitro* experimental viability data in single exposure from two human liver cell lines, HepG2 and HepaRG, were measured and used to optimize the VCBA, subsequently repeated exposure simulations were run. Amiodarone was then tested and simulations were performed under repeated exposure conditions in HepaRG. The results show that the VCBA can adequately predict repeated exposure experiments in liver cell lines. The refined VCBA model can be used not only to support the design of long term *in vitro* experiments but also practical applications in risk assessment. Our model is a step towards the development of *in silico* predictive approaches to replace, refine, and reduce the *in vivo* repeated dose systemic toxicity studies in the assessment of human safety.

1. Introduction

In order to reduce and eventually replace the use of animals for predicting toxicity in humans, models based on concentrations that cause effects *in vitro* will have to be developed, taking into consideration both toxicokinetics (TK) and toxicodynamics (TD). The characterisation of the concentration that produces an effect (whether this is a perturbation of a molecular pathway or an apical toxic endpoint) is necessary at two levels: first, for *in vitro* experiments since "nominal" concentrations do not correspond to real concentrations to which cells are exposed (Gülden et al., 2001; Groothuis et al., 2015); and, second, for extrapolating a dose for human toxicity assessment, since for the assessment of a hazard of a chemical compound, we need to know the true concentration in the target organ. Normally, concentration-response curves in *in vitro* experiments represent the total amount of substance added to a microtiter-plate well and not the dissolved (free) concentration that corresponds to a bioavailable fraction, which could induce an effect (Groothuis et al., 2015; Kramer et al., 2015; Hamon et al., 2015). However, these curves (often characterized by their potency parameters, *e.g.* IC50 values) do not properly reflect the actual concentrations, which induce an effect at the site of action of the chemical in the cell (particular receptor or enzyme). One possible improvement, already suggested and demonstrated by several research groups (Gülden and Seibert, 2003; Heringa et al., 2004; DeBruyn and Gobas, 2007; Kramer, 2010), would be to design *in vitro* experiments taking into account the toxic (but bioavailable) concentration in the medium which corresponds to the free dissolved concentration. In order to introduce a correction within the *in vitro* experimental set up our group previously developed the Virtual Cell Based Assay (VCBA) model (Zaldívar et al., 2010; Zaldivar Comenges et al., 2011; Zaldivar Comenges et al., in press), which can be considered an integrated modelling approach to improve the characterisation and analysis of *in vitro* cell-based assay data.

The VCBA was originally built using the Matlab platform and was applied to study the toxicological effects of chemicals on cells, as assessed by High Throughput Screening (HTS) and High Content Imaging (HCI) using single dose exposure conditions (Zaldívar et al., 2010; Zaldivar Comenges et al., 2011; Zaldívar et al., 2012). The model consists of ordinary differential equations whose solution allows the calculation of the dissolved concentration of a chemical over time, both in the plate and in the cells. The mathematical modelling of HTS and HCI experiments serves not only to predict experimental results (*e.g.* cell viability) but also to simulate the dynamics of several processes that are not easily measurable but which can be of toxicological relevance. These processes include chemical losses due to evaporation or adsorption onto plastic, as well as the effects of chemicals on cell growth and survival.

The integrated modelling approach of the VCBA thus consists of:

- A fate and transport model
- A cell partitioning model

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– A cell growth and division model
– A toxicity and effect model

This mathematical model takes into account the fate of a compound in the *in vitro* cell-based model, based on the partitioning between (i) the plastic wall, (ii) headspace, (iii) serum proteins, (iv) lipids, and potentially the compound dynamics within the cell. This is driven by a series of dynamic mass balance equations based on the compound physico-chemical properties and partitioning. The gas phase was included to allow, in a future version of the model, the possible losses and cross contamination between the 96 wells in the TC plates. The model is coupled with a cell growth model and a toxic effects model (Zaldivar Comenges et al., 2011). The cell growth model is a typical population model, described in terms of continuous ordinary differential equations, which ignores population structure by treating all individual cells as identical. Matrix population models (Caswell, 1989) integrate population dynamics and population structure and they are very useful when the life cycle is described in terms of size classes or age classes. In the case of the HepG2 cell line model, the appropriate description corresponds to a four stage-based approach, each stage corresponding to one of the four cell cycle phases: G1, S, G2, and M (Hartwell and Weinert, 1989). On the other hand the HepaRG cell line does not proliferate (it remains in phase G1). Furthermore the VCBA takes into account the Dynamic Energy Budget (DEB) approach, by Kooijman and van Haren (1990) showing changes in lipid contents and size of the organisms. The introduction of DEB models into matrix population models was already used by Lopes et al. (2005), Klanjscek et al. (2006); and Billoir et al. (2007). Following this approach, the chemical is taken up by the cell and it partitions instantaneously over three compartments: one aqueous fraction and two non-aqueous fractions: structural component (proteins) and the energy reserves (lipids). The direct toxic effects of a chemical concentration were related to the internal (intracellular) concentration of the toxicant with the no-effect concentration (NEC) for survival, and the kr, the killing rate of the toxicant (Zaldivar Comenges et al., in press). The combined model enables simulations of the true concentrations causing biological perturbations in cells, over time, given the nominal concentrations applied in a microtiter plate well.

In order to simulate the long-term (repeat dose) effects of chemicals, there is a need to develop a tool which can predict, in an efficient and reliable way, the intracellular concentration within the cell after multiple exposures. The following steps were applied in order to perform the repeated exposure simulations. First, for caffeine, *in vitro* experimental data from two human liver cell lines, HepG2 and HepaRG, were obtained. Second, the single exposure experimental cell viability data were used to optimize the VCBA (kr and NEC values). The third step after model optimisation was to simulate the time-dependent response and compare the predicted results with *in vitro* data found in literature or achieved experimentally in house; this can be considered as a validation step of the VCBA for caffeine. In the fourth step, using HepaRG cells, concentration response curves were tested experimentally and simulations for amiodarone under repeated exposure conditions were performed.

Thus, the VCBA was implemented into a KNIME workflow as described in Fig. 1, and an extension was made to simulate the repeated exposure, as illustrated in Fig. 2.

We describe the development of an open source VCBA methodology for simulating the long-term (repeat dose) toxic effects of chemicals, including substances found in cosmetics and personal care products, in *in vitro* (liver cell) systems. The approach is based on the previously developed VCBA model (Migita et al., 2010; Zaldivar Comenges et al., 2011; Zaldívar et al., 2012) which is re-coded and re-implemented in the open-source KNIME platform. The usefulness of this tool in simulating the repeated dose toxicity of selected compounds is illustrated with reference to two case study compounds: caffeine and amiodarone.

2. Materials and methods

2.1. Chemicals and supplies

HepaRG cells were obtained from Biopredic International (Rennes, France) and stored in liquid nitrogen. William's E medium, L-glutamine, penicillin/streptomycin and trypsin-EDTA were purchased from Invitrogen (San Giuliano Milanese, Italy). HyClone Fetalclone III serum was from Thermo Scientific (Pittsburgh, USA). Caffeine (58-08-2; purity set with HPLC was 99% as reported from supplier), bovine insulin, hydrocortisone hemisuccinate, and amiodarone hydrochloride (19774-82-4; purity by TLC > 98% as reported from supplier) were purchased from Sigma-Aldrich (Milan, Italy). Tissue culture treated 96 well clear bottom black polystyrene microplates were acquired from Corning (Pero, Italy). Fluorescence staining was performed with Hoechst 33342 (Invitrogen).

2.2. Cell culture and differentiation

Differentiated HepaRG cells exhibit many characteristics of primary human hepatocytes, including morphology and expression of key metabolic enzymes, nuclear receptors, and drug transporters (*e.g.* morphology) and are metabolically competent, thus express relevant phase I and II enzymes. HepaRG cells were cultured in William's E medium supplemented with 10% HyClone Fetalclone III serum, 1% L-glutamine, 1% penicillin/streptomycin, 5 μg/mL insulin and 50 μM hydrocortisone hemisuccinate. The cells at passage 18 were seeded at a density of 4×10^6 cells in 150 cm^2 flasks and the medium was refreshed every two days. After two weeks, 1.7% DMSO was added to favour the cells

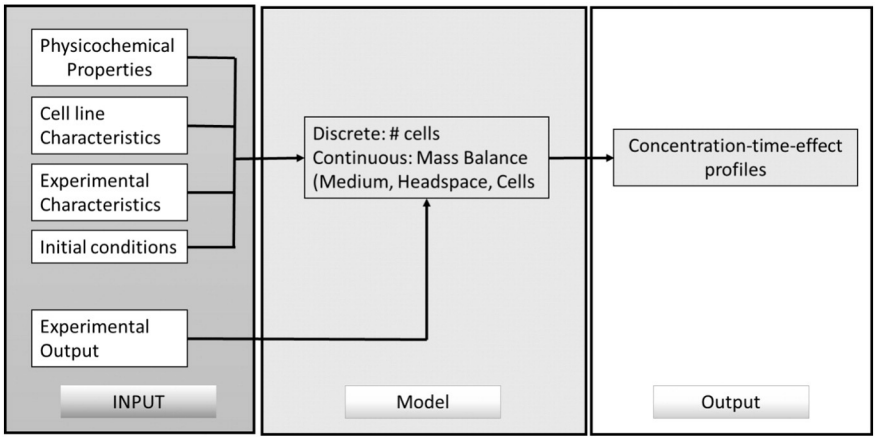

Fig. 1. A graphical representation of the general setup of the automated VCBA implemented into KNIME platform; which consists of three zones: input, model, and output.

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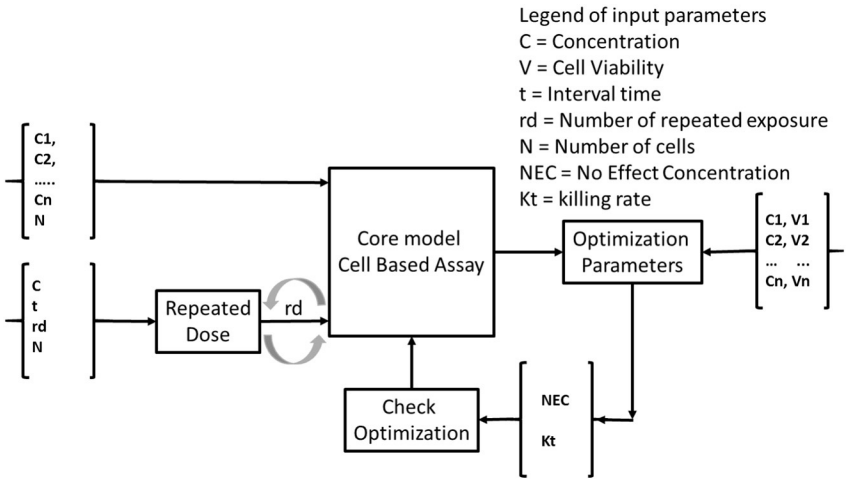

Fig. 2. Setup of the automated VCBA for repeated exposure. The model is set up to start with a range of concentrations in single exposure to optimize the NEC and kr. After which, the model will simulate the repeated exposure based on the starting number of cells present at time zero, the nominal concentrations applied, the number of repeated doses and experimental time point selected.

undergoing complete hepatocyte differentiation. Finally, the differentiated cells were gently harvested with trypsin-EDTA and seeded into 96-well clear bottom black plates at a density of 50,000 cells/well using the Starlet Hamilton platform (Agrate Brianza, Italy). Before exposing the cells to amiodarone or caffeine, isolated hepatocytes were incubated for 72 h in medium without DMSO at 37 °C, 5% CO_2, and 100% humidity.

The human hepatoma derived HepG2 cell line consists of adherent epithelial-like cells growing as monolayers and in small aggregates. The seeding density (passage 12) was of $15 * 10^3$ cells/well, cells were incubated at 37 °C in a humidified 5% CO2 incubator for a period of 24 h prior to testing (Clothier et al., 2013).

2.3. Repeated exposure of HepaRG to selected chemicals

For the validation of the model, caffeine and amiodarone were used as test compounds. Concentrations of caffeine, ranging from 0.195 to 75 mM (24 h exposure, single exposure) or 0.0355 mM to 9.1 mM for the repeated exposure were tested at 24 h, 48 h, 72 h, and 96 h, using 5 or 3 wells for each concentration (technical replicates) respectively. Amiodarone was tested in HepaRG at eleven concentrations (between 0 and 50 μM) with three biological replicates. The exposure intervals were: 24 h, 72 h, 7 days, 14 days; at these time points measurements were performed. The experimental protocols were similar to those previously reported in Mennecozzi et al. (2011) and in Zaldívar et al. (2010). Chemicals were initially solubilized in 100% DMSO and then diluted in culturing medium with 5% HyClone Fetalclone III serum to obtain a final concentration of DMSO of 0.1%. The range of chemical concentrations, obtained by performing 1:2 serial dilutions, was tested in isolated hepatocytes from 2-week-old confluent cultures. Cells treated with only 0.1% of DMSO were included as negative controls (4 replicates per plate).

2.4. Cell staining and high content imaging

At selected time-points (24 h, 72 h, 7 days, and 14 days), treated HepaRG cells were stained with Hoechst 33342 for 30 min. Live cell imaging was performed using Cellomics ArrayScan vTi (Thermo Scientific, Pittsburgh, PA, USA). A 10× objective was used to collect 10 image fields per well (starting at the centre of a well) with a XF93 filter (Omega Optical, Brattleboro, VT). Cell count, nuclear area and intensity (Hoechst 33342 dye), were measured using Cytotoxicity Bioapplications v.4 from Cellomics Scan Software (Thermo Scientific, Pittsburgh, PA, USA). The HCI data obtained after cellular exposure were plotted against tested concentrations.

2.5. Viability in HepG2 assessed using MTT assay

HepG2 (human liver carcinoma cells) are adherent, epithelial-like cells growing as monolayers and in small aggregates. *In vitro* data generated in the context of the EU FP6 project ACuteTox were used (Clothier et al., 2013). The caffeine concentration response curves (0.0343–75 mM) were generated by using the MTT assay. In brief, HepG2 cells were cultured in 96-well plates. After 24 h treatment with caffeine, cells were incubated with the 100 μL MTT solution (0.5 μg/mL) for 2 h, then washed with 100 μL of pre-warmed phosphate buffered saline (PBS). The formazan formed in the cells was solubilized in DMSO and measured colorimetrically. The amount of formazan produced was then quantified using a simple colorimetric assay. The results were read in a multi-well scanning spectrophotometer (ELISA reader). Cytotoxicity was expressed as inhibitory concentration (ICX) of a chemical resulting in an X% reduction of the cell number/viability, as compared to the untreated control. Three biological triplicates were performed.

2.6. The Virtual Cell Based Assay (VCBA)

The VCBA model (Zaldívar et al., 2010; Zaldivar Comenges et al., 2011; Zaldívar et al., 2012) was extended to include two different cell lines, HepG2 and HepaRG. Briefly, the VCBA model integrates: 1) the *in vitro* fate and transport model. The fate and transport model calculates the time-variable chemical concentration in the medium as well as in the headspace. It takes into account a series of processes including evaporation, partitioning of chemicals from the dissolved phase to serum proteins and lipids, adsorption onto the plastic, degradation, and decomposition. 2) The cell growth and division model. The cell growth and division model is based on a four stage based approach (Gérard and Goldbeter, 2009), with each stage corresponding to one of the four cell cycle phases: G1, S, G2 and M (Zaldívar et al., 2010). The following cell lines were used: HepaRG and HepG2. For HepaRG no proliferation occurs, only G1 phase is present, and for HepG2 see Tables 2 & 3, results Section. 3) The cell partitioning model. In the present work liver cells were introduced into the model. This model was based on the assumption that once the chemical is taken up by the cell, a partitioning occurs between three compartments: one aqueous fraction and two non-aqueous fractions corresponding to structural components (proteins) and energy resources (lipids). 4) The

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toxicodynamics model. The direct effects of a chemical concentration, C, on cell dynamics (survival/mortality) are expressed by using the killing rate, kr, and the no effect concentration, NEC (Lopes et al., 2005; Billoir et al., 2007). 5) The experimental set up. This takes into account the surface, area, size and shape of the well or well-plate.

To run the model a series of input parameters are needed. Some can be found in the literature, by applying QSARs, or if necessary to obtain them experimentally. Parameters that are not directly measurable, such as no effect concentration (NEC) and killing rate (kr), can be calculated from the *in vitro* HTS experiments and can be computed by optimisation using an algorithm that minimizes the squares of the differences between the experimental cell viability and the viability predicted (simulated) by the model. The model calculates the NEC and kr, using the following function:

$$error = \sum_{i=1}^{n^{o}\,\mathrm{exp}} \left(Viability_{\mathrm{exp}} - Viability_{sim}\right)^{2} \quad (1)$$

where $Viability_{sim}$ and $Viability_{exp}$ represent the simulated and experimental cell viabilities, respectively.

The VCBA was implemented in an open source KNIME platform using the R program (both freely available). KNIME is a user-friendly graphical workbench for the analysis process such as data access, data transformation, investigation, visualization and reporting (http://www.knime.org/). With the Optimization Parameters tab in the VCBA KNIME workflow the model was optimised in an automated way, as described above. The core model performs a simulation of the viability for each of the chemical concentrations. The error function (Eq. (1)) calculates the error from the values of NEC and kr. This error function is minimized using the optimization function of R, a graphical representation is created to see the performance of the model optimization (Fig. 6).

3. Results

The following steps were applied in order to perform the repeated exposure simulations. First, for caffeine, *in vitro* experimental data from two human liver cell lines, HepG2 and HepaRG, were obtained. Second, the single exposure experimental cell viability data were used to optimize the VCBA model (kr and NEC values). The third step after model optimisation was to simulate the time-dependent response and compare the predicted results with *in vitro* data found in literature or achieved experimentally in house; this can be considered as a validation step of the VCBA model for caffeine. In the fourth step, amiodarone under repeated exposure conditions in HepaRG was applied for simulations.

3.1. In vitro viability data from HepG2 cells

The results show that caffeine exposure to HepG2 for 24 h resulted in a statistically significant reduction of cell viability at the two highest concentrations of 25 mM and 75 mM. The concentration-response curve (Fig. 3) obtained was used to optimise the VCBA model in single exposure mode for the HepG2 cell line.

3.2. In vitro viability data from HepaRG cells - single exposure experiment

The results show that HepaRG cells exposed to caffeine for 24 h undergo a statistically significant reduction of cell viability at concentrations higher than 3 mM. This concentration-response curve (Fig. 4) was used to optimize the VCBA model in single exposure mode for the HepaRG cell line.

3.3. In vitro viability data from HepaRG - repeated exposure experiment

The results (Fig. 5) show that HepaRG cells exposed to caffeine (0–9 mM) repeatedly show a statistically significant decrease in cell viability already at 48 h, but a drastic decrease is found starting from 2.2 mM at 72 h and 96 h.

3.4. Simulation of the fate of caffeine and amiodarone in HepaRG

Table 1 reports the simulated partitioning, as percent concentration [%] of both compound tested in HepaRG at 24h, achieved from the fate model. The medium represents the total starting amount (100%) of 0.155 mM.

The results show that caffeine is more water soluble - up to 94% is in the dissolved state that could enter the cell. On the contrary, amiodarone is more lipophilic - 85% of the chemical binds to lipids and almost 4% migrates into the plastic of the well.

3.5. Validation of VCBA model for repeated exposure to caffeine

HepG2 data taken from the literature (Scheers et al., 2001), were represented as cell viability in % versus the control (set to be the PI_{50} at 24 h, PI_{50} is the concentration of compound needed to reduce the total protein content to 50% after 24 h of treatment). Following optimization of the single-exposure VCBA model in a single exposure mode, these data were used to compare the VCBA model simulations under repeated exposure conditions. The data show fast cell death at a concentration of 4.67 mM caffeine within two weeks of exposure (Fig. 7a).

3.6. Simulation in single exposure mode using selected in vitro hepatic cell lines.

The VCBA growth model was set to only G1 phase for HepaRG cells, since this cell line is known not to proliferate; Tables 2 & 3 report the cell specific input parameter to run the VCBA. On the one hand it has been

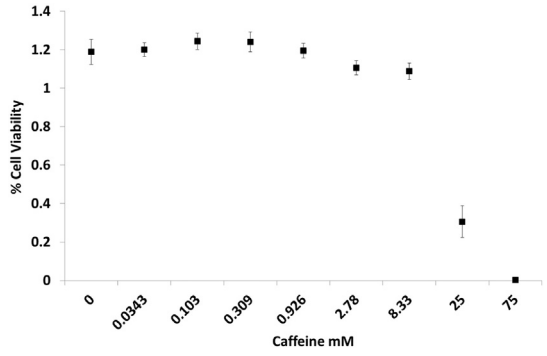

Fig. 3. 24 h Caffeine effect on cell viability of HepG2 cell line (mean of three biological replicates), (extract from Clothier et al., 2013).

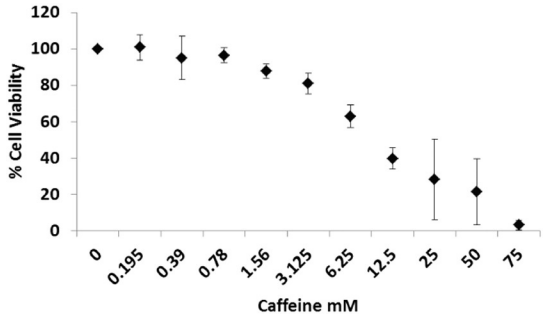

Fig. 4. 24 h Caffeine effect on cell viability of HepaRG cell line (mean of three biological replicates).

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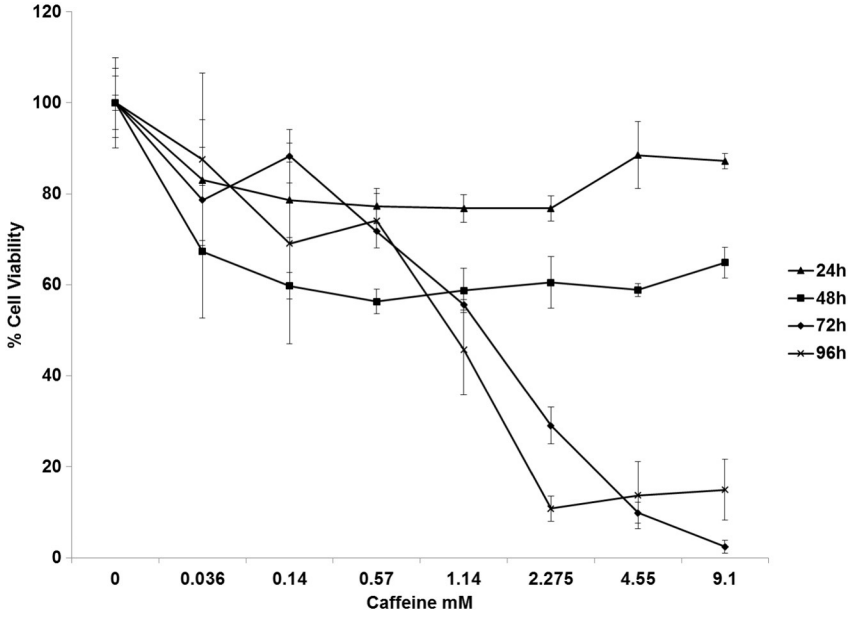

Fig. 5. Effects of repeated exposure of caffeine on HepaRG cell viability (mean of three technical replicates).

shown that HepG2 cells are able to metabolise xenobiotic compounds leading to toxic effects, including genotoxicity, oxidative stress and mitochondrial dysfunction (Hewitt and Hewitt, 2004; Knasmuller et al., 2004; O'Brien and Haskins, 2007; Schoonen et al., 2009). On the other hand, it is undeniable that the HepG2 cells have lost some of the liver-specific functions due to immortalisation, in particular the phase I drug-metabolising enzymes such as CYP2E1 (Boess et al., 2003; Wilkening and Bader, 2003). The VCBA for this cell line takes into account the growth (cell cycle) model: G1 – S – G2 – M. The data on the duration (h) in each stage for HepG2 was taken from Migita et al. (2010), HepG2 = [51.4, 33.2, 10.7, 4.7] (h), whereas the total duration was let as an optimization parameter for HepG2. Mortalities were obtained from Kudryavtsev et al. (2004), zi = [0.005, 0.005, 0.04, 0.04] (h^{-1}). F was also another optimization parameter, which expressed as a function of cell density

$$F = F_0 \cdot \exp\left(-\sum_{i=1}^{4} N_i/\rho\right) \quad (2)$$

for the case of HepG2. Overview of the HepG2 cell specific parameter are listed in Table 2 and values to describe the cell growth model is reported in Table 3.

3.7. Optimization parameter and model calibration in KNIME platform

With the optimization parameters step, as part of the KNIME platform, we set up: (i) No Effect Concentration (NEC) for survival and (ii) the Killing Rate (kr) of the toxicant. The model was fitted to the experimental value in order to minimise the error, and achieve the optimised NEC and kr values. To validate the model; the results achieved were compared with the toxicity experimental data performed in repeated exposure. For caffeine, the VCBA was optimised to minimise the error in model prediction of the no effect concentration and killing rate, using experimental values for two cell lines, HepG2 (Fig. 6A) and HepaRG (Fig. 6B), in single exposure mode. This resulted in the following optimised values of NEC and kr for the two cell lines exposed to caffeine:

HepG2, NEC = 0, kr = 1.1107
HepaRG, NEC = 0, kr = 0.222

3.8. Validation of the Virtual Cell Based Assay for repeated exposure to caffeine

After the optimisation step of the VCBA performed by using *in vitro* data from single exposure experiments, the model was run using the

Table 1
VCBA estimates in % of the chemical fate in the different compartments of the model, using HepaRG cells.

	Medium[a]	Headspace	Dissolved	Proteins	Lipids	Plastic
Caffeine						
0.155 mM	100%	1.32E−07	93.95	5.94	0.00122	0.0076
Amiodarone						
0.155 mM	100%	1.11E−13	0.0018	10.67	85.44	3.87

[a] Medium represents the total starting amount (100%) of 0.155 mM.

Table 2
VCBA parameters for HepaRG and HepG2 cell lines.

Parameter type	Abbreviation used in the model	HepaRG	HepG2	Units
Mass fraction of compartment f_x (aq-*aqueous*, l-lipids, p-proteins)	f_{aq}	0.72 (72%)	0.70 (70%)	% weight
	f_L	0.012 (1.2%)	0.06 (6%)	
	f_P	0.268 (26.8%) (JRC In house data)	0.24 (24%) (JRC In house data)	
Initial cell radius	r_o	—	$8.3057 * 10^{-6}$	m
Final cell radius	r_∞	—	$6.592 * 10^{-6}$	m
von Bertalanffy's growth rate	α_G	—	$1.2687 * 10^{-5}$ (Zaldívar et al., 2010, Zaldivar Comenges et al., 2011)	s^{-1}
Density: protein	$P_{P,}$	1350	1350	g/L
Lipid	$P_{L,}$	900	900	g/L
Aqueous	$P_{aq,}$	1000	1000	g/L
Density cell type	P	1072	1085 (Zaldívar et al., 2010, Zaldivar Comenges et al., 2011)	g/L
Serum in medium		10	5	%

Table 3
VCBA parameters for cell growth in HepaRG and HepG2 cells.

HepG2	G1	S	G2	M
Duration (h)	51.4	33.03	10.7	4.7
Mortality (h^{-1})	5.00E−03	5.00E−03	4.00E−02	4.00E−02
Volume (m^3)	1.73E−15	2.40E−15	2.40E−15	2.40E−15
Mass (g)	2.08E−09	2.40E−09	2.40E−09	2.40E−09
Initial cell population (%)	0.514 (51.4%)	0.332 (33.2)	0.107 (10.7%)	0.0469 (4.69%)
Cell division rate	3.81E + 00			
HepaRG	G1	–	–	–
Duration (h)	849			
Mortality (h^{-1})	1.19E−15			
Volume (m3)	1.67E−15			
Mass (g)	1.79E−9			
Initial cell population (%)	1 (100%)			
Cell division rate	0			

optimised NEC and kr values (NEC = 0, kr = 1.1107). We ran the model for repeated exposure in HepG2, selecting an interval time of 1 week. The VCBA model simulations for HepG2 were compared with *in vitro* data found in literature (Scheers et al., 2001), whereas for HepaRG cells, the simulations were compared with HTS data generated in house. The simulated cell viability-time curves are depicted in Fig. 7A and B, along with the experimental data.

3.9. Simulation of repeated exposure effects of amiodarone

The viability of HepaRG cells exposed to amiodarone was tested in HTS. The NEC and kr were optimised by applying the 24 h experimental data (as done with caffeine), the NEC and kr values achieved were 0.000692 and 0.199, respectively. These values were used for simulations of the repeated exposure, shown in Fig. 8 (lines), the results are reported for 5 concentrations out of the eleven tested (to keep the graphical representation easy to read). For amiodarone the simulation was based on a 14 days exposure, covering the treated exposure time intervals. The simulations were plotted (Fig. 8) with the experimental results (fill mark), showing a closer fit to the experimental data at the highest tested concentration. This could be an indication that the partitioning coefficients used in the present model are more reliable for simulation of high concentrations.

4. Discussion

In the context of replacing the use of animals in toxicity testing, there is a need to predict human *in vivo* toxic doses from concentrations that cause toxicological effects in relevant *in vitro* systems. The characterisation of the concentration that produces an effect (whether this is a perturbation of a molecular pathway or an apical toxic endpoint) is necessary at two levels: first, for the correction of *in vitro* concentration response curves, since "nominal" concentrations do not represent the real concentration experienced by the cell; and, second, in extrapolating *in vitro* effects to humans, since the true concentration experienced by cells within the target organ is more relevant for human toxicity assessment.

In order to address the first issue (refinement of *in vitro* experiments) the Virtual Cell Based Assay (VCBA) was developed (Zaldivar Comenges et al., in press). The VCBA is a mathematical model consisting of ordinary differential equations the solution of which allows the calculation of the dissolved concentration of a chemical in cell culture over time as well as the internal concentration in the cells, which is currently applicable to a range of cell lines (3T3c Balbc, HepG2, HepaRG, A459, and cardiomyocytes). As described in detail in Zaldivar Comenges et al. (in press), the VCBA comprises of five interconnected models: i) a fate and transport model. ii) a cell partitioning model. iii) a cell growth and division model. iv) a toxicity and effects model. v) the experimental set up.

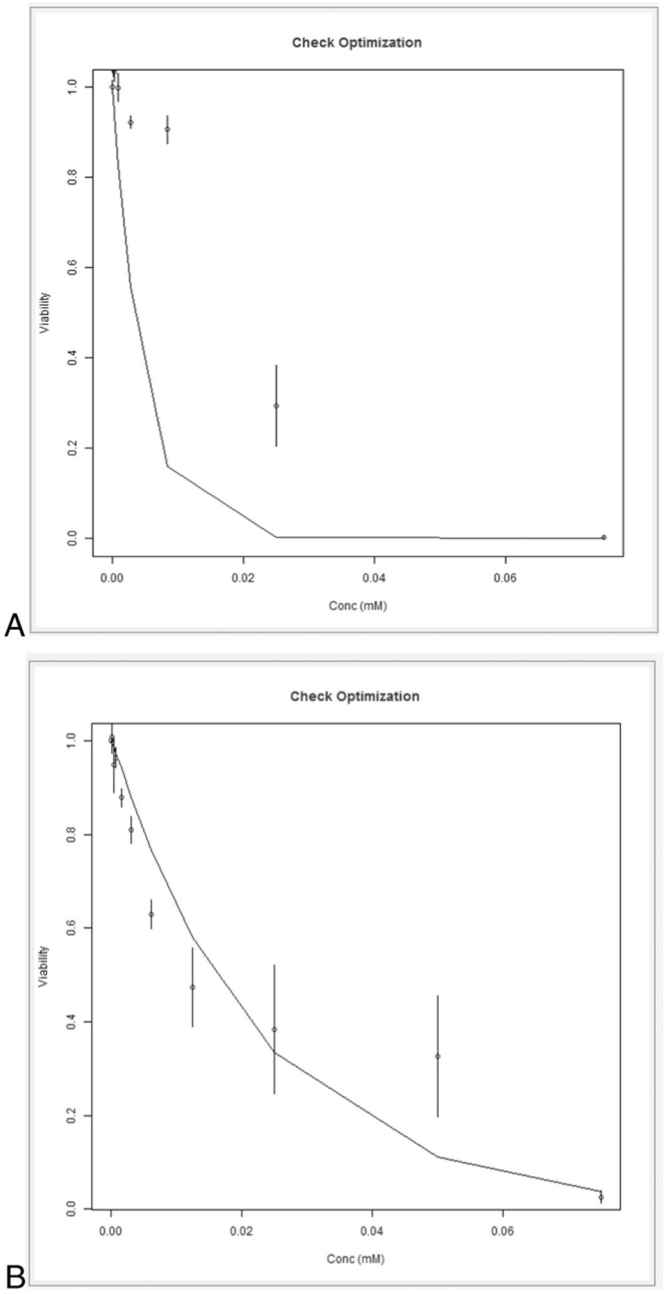

Fig. 6. A. VCBA optimisation using *in vitro* experimental data obtained by exposing HepG2 cells to caffeine. B. VCBA model optimisation using *in vitro* experimental data obtained in HepaRG exposed to caffeine (graphs are output from KNIME workflow).

In the present study, we extended the applicability of the VCBA to simulate single exposure effects (Zaldívar et al., 2010; Zaldivar Comenges et al., 2011; Zaldívar et al., 2012) to repeated exposure effects. The following steps were taken to simulate the repeated exposure scenario: i) for caffeine, *in vitro* experimental concentration response measuring viability from two types of human liver cell lines, HepG2 and HepaRG, were obtained. The concentration response curve range was selected to be in line with the available experimental data (Clothier et al., 2013). However, one needs to take into account that in some cell types hyperosmotic stress occurring at high concentrations could lead to cytoskeleton rearrangement and cell cycle arrest as well as protein and DNA damage (Arsenijevic et al., 2013), which eventually

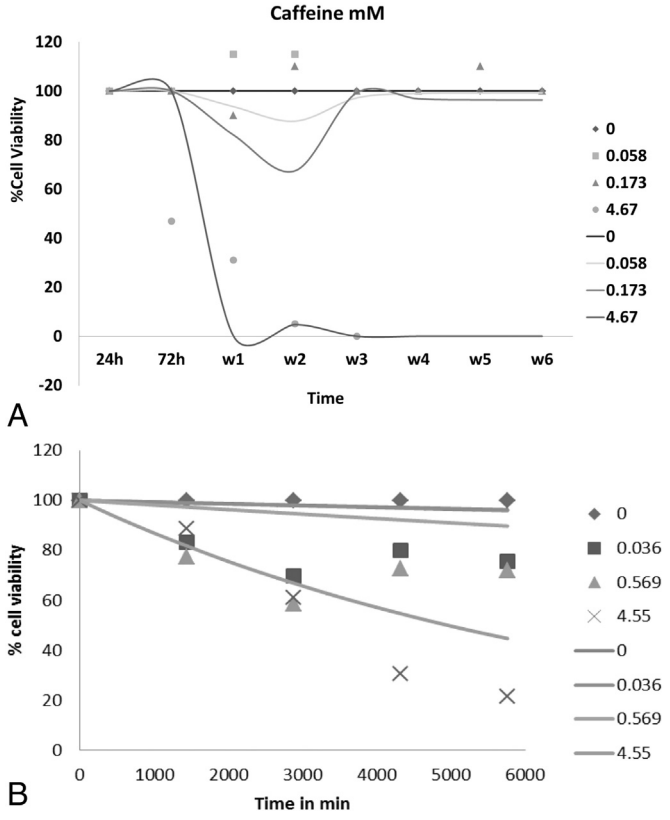

Fig. 7. A. VCBA simulations (lines) at corresponding concentrations used *in vitro* in HepG2 cells exposed to caffeine (mM) in time (h, hours, w, week). B. VCBA time response simulations (lines) at corresponding concentrations used *in vitro* in HepaRG cells exposed to caffeine (mM) in time (min).

could lead to apoptosis (Lang et al., 2006). The strength of this effect on HepaRG cells should be studied further. ii.) Single experimental cell viability data were used to optimize the VCBA. iii.) After optimisation of the model parameters NEC and kr, these values were used to simulate the time-dependent response and compare the predicted results with *in vitro* data found in literature for HepG2 and obtained in the laboratory for HepaRG. We could consider this as a validation step of the caffeine based VCBA. The same approach was used for amiodarone applied only to HepaRG cell line. NEC and kr were achieved and tested time-dependent response curves for several amiodarone concentrations under repeated exposure conditions. The simulated repeated exposure showed with caffeine has shown (in HepaRG) a slight overestimation

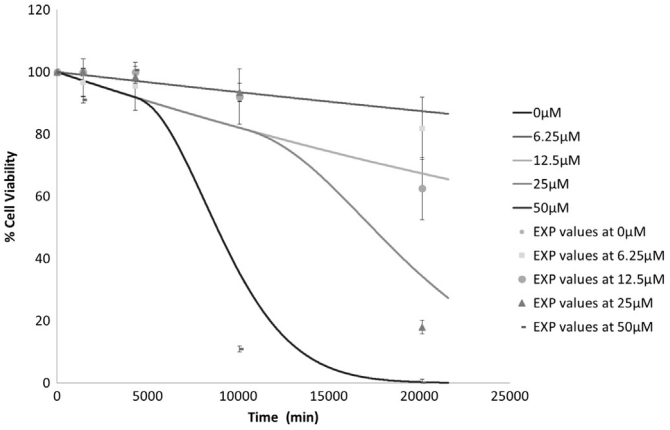

Fig. 8. VCBA simulations (lines) at corresponding concentrations used *in vitro* in HepaRG cells exposed to amiodarone (μM), simulation line 0 and 6.25 μM are overlapping.

of the cell viability compared to the *in vitro* results and only above the concentration of 4.55 mM the model had same trend as the *in vitro* results. This could implicate that the caffeine VCBA model is less well calibrated for lower concentrations. On the other hand, in case of HepaRG cells that were exposed repeatedly to amiodarone, the simulations were much more reliable at the different concentrations tested *in vitro*.

The main motivation for developing the VCBA model was to provide a means of simulating the real concentrations that reaches and perturbs the cells in *in vitro* experiments, in single (acute) or repeat (chronic) exposure. Since the mathematical model takes *in vitro* processes (biokinetics) into account (*e.g.* losses by evaporation and binding to the plastic wells), the actual concentrations should reflect more realistic and reliable dose metrics of chemical toxicity than the nominal concentrations applied in *in vitro* systems. These simulated concentrations can thus be used in the design of follow-up *in vitro* experiments and to improve, for instance, the reliability of *in vitro* to *in vivo* extrapolation for risk assessment purposes. Also, intracellular total concentrations (or even intracellular free – unbound – concentrations) might provide a better dose metric than the extracellular concentration in the medium (total or free). Future work should aim to compare the free (unbound) extracellular concentrations ($IC_{50\ free}$) with intracellular concentrations ($IC_{50\ int}$), and check which ones are more predictive in extrapolating *in vitro* to *in vivo* effects. Therefore, there is a need to enrich the database with additional experimental results coming from the usage of different chemicals, cell lines, treatment protocols *etc*., as well as refinement of the VCBA to take into account more complex systems (*e.g.*, bi-compartmental systems, micro-chips) (Armitage et al., 2014; Crean et al., 2014; Truisi et al., 2015; Wilmes et al., 2013).

Other VCBA like models have also been developed to predict repeated dose. For example, Kramer (2010) used Rainbow trout (*Oncorhynchus mykiss*) cell lines RTL-W1 and RTgill-W1 to develop a five-compartment model [air, medium, protein, plastic, cell] to be able to simulate cell viability effects following repeated exposure to three compounds (benzo(a)pyrene, 1,2-dichlorobenzene, 1,2,4-trichlorobenzene). Two-compartment VCBA models [medium and cell] plus a sub compartment for metabolic clearance for primary rat hepatocytes, primary human hepatocytes, and the HepaRG cell line, were developed to simulate single and repeated exposure to Chlorpromazine (Broeders et al., 2014). A recent three-compartment [cell, medium, protein] VCBA model was developed for ibuprofen to simulate repeated exposure and cell viability effects in primary rat hepatocytes, primary human hepatocytes, and HepaRG cell line (Truisi et al., 2015). Other models that could probably be adapted to repeated exposure conditions are those developed by Armitage et al. (2014) and Stadnicka-Michalak et al. (2014).

To simplify and automate its use the VCBA was implemented as an open source tool in the KNIME platform. Future human safety assessments will rely increasingly on the use of multi-scale models, such as Physiologically-Based Kinetic/Dynamic (PBK/D) models and Virtual Cell Based Assay (VCBA) models, implemented through a combination of computational tools, in order to perform extrapolations such as *in vitro* to *in vivo* extrapolation (IVIVE). These biologically-based models will be coupled with chemistry-based prediction models that also automate the generation of key input parameters such as physicochemical properties. The development of automated software tools is an important step in harmonising and expediting the chemical safety assessment process.

We believe that the *in silico* tool described in this paper (VCBA in repeat exposure mode) is a step towards the development of predictive approaches that could eventually replace, refine and reduce the need for *in vivo* repeated dose systemic toxicity studies in the assessment of human safety.

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